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(54) Title: GALECTIN-8 AND GALECTIN-8-LIKE PROTEINS AND DNA MOLECULES CODING THEREFOR

(57) Abstract

The invention relates to a new mammalian S-type lectin, termed galectin-8, and to galectin-8-like proteins, to fragments thereof, to DNA molecules coding therefor and to pharmaceutical compositions comprising said proteins. Galectin-8, a widely expressed protein of 35 kDa is shown to be implicated in regulation of cell growth, particularly in inhibition of cell proliferation.

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GALECTIN-8 AND GALECTIN-8-LIKE PROTEINS AND DNA MOLECULES CODING THEREFOR

FIELD OF THE INVENTION

The present invention is generally in the field of mammalian S-type lectin proteins, now designated galectins, which are thiol-dependent and specifically bind β-galactoside residues.

More specifically, the present invention relates to a new S-type mammalian lectin, termed hereinafter as "galectin-8", and to galectin-8-like proteins, to DNA molecules coding therefor and to antibodies raised against said proteins. The invention further relates to pharmaceutical compositions comprising said proteins for the purpose of cell growth regulation in general, and more particularly for inhibition of cell proliferation and for treatment of tumors.

BACKGROUND OF THE INVENTION

Lectins are involved in a wide variety of cellular functions, many of which are related to their only common feature, the ability to bind carbohydrates specifically and reversibly, and to agglutinate cells [reviewed in (1)]. Animal lectins are classified as C-lectins, which are Ca^{2+}-dependent and are structurally related to the asialoglycoprotein receptor, and galectins, previously known as S-type lectins, which are thiol-dependent and specifically bind β-galactoside residues. In mammals, four galectin types have been sequenced and characterized, and there is evidence for the existence of other relatives (2,3). All known members of this family lack a signal peptide, are found in the cytosol, and are isolated as soluble proteins. However, there is evidence that some members are externalized by an atypical secretory mechanism.

Galectins require fulfillment of two criteria: affinity for β-galactosides and significant sequence similarity in the carbohydrate recognition domain (CRD) (4), the relevant amino

acids residues of which have been determined by X-ray crystallography (5). Galectin-1 and -2 are homodimers with subunit molecular weight of ≈14 kDa, that are not subjected to post-translational modifications (6). Galectin-1 is found in the extracellular matrix and has been shown to interact with laminin (7). The function of galectin-1 and -2 is not yet fully understood, although there is evidence that they might be involved in regulation of cell growth (8); cell adhesion (7); cell transformation (9); and embryogenesis (10).

Larger galectins (galectin-3) (previously known as CBP-35, Mac-2, RL-29) do exist ((11) and references therein). These are monomeric 29-35 kDa mosaic proteins, composed of an N-terminal half made of tandem repeats characteristic of the collagen gene superfamily, and a C-terminal half homologous to galectins-1 and -2 (11). Galectin-3 also binds laminin, and is implicated as component of growth regulatory systems; mediator of cell-cell and cell-matrix interactions; modulator of immune response; marker of neoplastic transformation, and indicator for metastatic potential of melanoma cells.

Galectin-4 was cloned from rat intestine (12), and an homologous protein was cloned from nematode (13). Galectin-4 is a monomer with molecular mass of 36 kDa. It contains tandem domains of ≈140 amino-acids each, homologous to galectin-1 and -2, that are separated by a link region (12). The function of galectin-4 is presently unknown.

Galectins may functionally substitute each other. The absence of any major phenotypic abnormalities in mice carrying a null mutation in the gene encoding galectin-1, suggests that other protein(s), presumably galectin-3, are capable of functionally substituting for galectin-1, at least at early stages of embryogenesis.

It is an object of the present invention to provide the cloning of a cDNA encoding for a novel protein that we term galectin-8. Galectin-8 has the characteristic properties of other galectins (2,3), and it is structurally related (34% identity) to rat galectin-4 (12).

SUMMARY OF THE INVENTION

According to the present invention, a novel protein of 35 Kd which has the characteristic properties of galectins (S-type mammalian lectins) was cloned from rat liver cDNA expression library. This protein was originally called by us RL-30 protein. However, the nomenclature of S-type lectins has recently been changed to galectins (2). Since names for galectins 1-7 were already assigned (3), this new protein has now been named galectin-8, but it is to be understood that this is the same protein formerly called by us RL-30.

Thus, in one embodiment, the present invention provides a biologically active S-type lectin named galectin-8 and galectin-8-like proteins and fragments thereof selected from:

(i) the protein galectin-8 of the amino acid sequence depicted in Fig.1;

(ii) a protein having greater than about 80 percent similarity to all or part of the sequence of amino acid residues 1-316 depicted in Fig. 1;

(iii) a protein having greater than about 80 percent similarity to all or part of the sequence of amino acid residues 1-151 depicted in Fig. 1;

(iv) a protein having greater than about 80 percent similarity to all or part of the sequence of amino acid residues 152-316 depicted in Fig. 1;

(v) a protein of (i), (ii), (iii) or (iv) in which one or more amino acid residues have been added, deleted, replaced or chemically modified without substantially affecting the biological activity of the protein;

(vi) a biologically active fragment of (i) to (v); and

(vii) an homologous polypeptide to that of (i) to (vi) derived from another mammal and which has a similar biological activity to that of (i) to (vi).

In another embodiment, the present invention relates to an isolated DNA sequence encoding galectin-8 or a galectin-8-like protein.

By one embodiment, the isolated DNA sequence of the invention is one that encodes a polypeptide product of

prokaryotic or eukaryotic host expression, said product having all or part of the primary structural conformation of galectin-8 or of a galectin-8-like protein and having the biological activity of galectin-8.

The above DNA sequence of the invention may be any one of the group consisting of:

(i) a DNA molecule having a nucleotide sequence derived from the coding region of a native galectin-8 or galectin-8-like gene;

(ii) a DNA molecule capable of hybridization to the cDNA clones of (i) under moderately stringent conditions and which encodes biologically active galectin-8 or a galectin-8-like protein; and

(iii) a DNA molecule which differs, as a result of the degenerative nature of the genetic code, from the DNA sequences defined in (i) or (ii) and which encodes biologically active galectin-8 or a galectin-8-like protein.

By way of other embodiments, the above DNA sequence of the invention is one selected from:

(i) a DNA molecule comprising the coding nucleic acid sequence depicted in Fig. 1;

(ii) a DNA molecule having the nucleic acid sequence of (i) in which one or more codons has been added, replaced or deleted in a manner that the polypeptide encoded by said sequence essentially retains the same biological properties as the polypeptide encoded by an unaltered DNA sequence;

(iii) a DNA molecule encoding a polypeptide having an amino acid sequence of a polypeptide encoded by the DNA molecule of (i) or (ii) but which differs therefrom in view of the degenerative nature of the genetic code;

(iv) a DNA molecule having a coding nucleotide sequence, which is homologous to the DNA molecule of (i), (ii) or (iii), which is derived from a mammal other than rats and which encodes a polypeptide having a similar biological activity to that encoded by the sequences of (i), (ii) or (iii);

(v) a fragment of the coding sequence of (i)-(iv) which encodes a polypeptide which essentially retains the biological properties of the polypeptide encoded by the unfragmented DNA

molecule; and

(vi) a DNA molecule comprising the coding DNA sequence of a fragment of (i)-(v) and additional DNA sequences in the 3' and 5' ends.

In a further embodiment, the present invention relates to a recombinant DNA molecule comprising a coding sequence according to any of (i)-(iii) and (i)-(iv) above or a fragment thereof according to (v) or (vi) above.

The present invention also provides a recombinant expression vector comprising any one of the above-mentioned DNA molecules of the invention. Such a recombinant expression vector may be one capable of being expressed in prokaryotic or eukaryotic hosts, the vector containing, in addition to any one of the above galectin-8 or galectin-8-like protein encoding sequences, various other sequences such as, for example, those sequences that are known to be important for expression of the desired sequence and the maintenance and propagation of the vector in the host cell. Construction of such recombinant expression vectors is by way of x22any of the known procedures.

The present invention further provides a method for preparing galectin-8 or a galectin-8-like protein or a biologically active fragment thereof, comprising culturing a suitable host cell containing the above recombinant vector of the invention under conditions promoting expression.

The protein of the invention may be prepared, as noted above, by expression of a recombinant vector comprising a DNA sequence encoding the protein, or it may be isolated and purified from various mammalian tissues using standard procedures for protein extraction and purification. In such purification procedures there may be employed yet another aspect of the present invention, namely, antibodies which are immunoreactive with native or recombinant galectin-8 or with a galectin-8-like protein. Such antibodies may be applied in standard affinity chromatography methods to provide for the final purification steps of the galectin from various tissues. The preparation of the antibodies is by standard procedures using native or recombinant galectin-8 or a fragment thereof or a galectin-8-

like protein or a fragment thereof as antigen or immunogen to stimulate antibody production in suitable animals. Both polyclonal and monoclonal antibodies to galectin-8 are encompassed by the invention. These antibodies can be prepared by standard procedures well-known in the art.

The anti-galectin-8 antibodies of the invention may also be employed in an assay method for the detection of overexpression of galectin-8 in mammalian tissue, said method comprising applying an effective amount of the antibodies to a tissue or body fluid sample obtained from a mammal and determining the extent of antibody binding to the sample. In such an assay, standard procedures may be employed, such as, for example, ELISA assay procedures.

In addition, the present invention also provides pharmaceutical compositions comprising as active ingredient an effective amount of galectin-8 or of a mammalian galectin-8-like protein and a suitable diluent or carrier, in particular compositions for cell growth regulation, more specifically for the inhibition of cell proliferation, for example for the treatment of cancer.

In these above compositions the diluents or carriers may be any of those substances well known in the art for the preparation of pharmaceutical compositions, and likewise the compositions may be prepared by standard procedures. Actual dosages and modes of administration of the above compositions are to be determined by skilled professionals.

DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts the cDNA sequence of galectin-8 and deduced protein sequence. The cDNA sequence of 1247 base pairs (bp) contains an open reading frame from 121-1069 bp, which encodes for a protein of 316 amino acids.

Fig. 2 shows that galectin-8 encodes for a galectin with two homologous carbohydrate-binding regions. A schematic structure of galectin-8 is presented (top). Each box represents a putative carbohydrate-binding domain, linked by a 32 amino acid long peptide. Shown are invariant amino acids preserved in most

galectins analyzed so far. The Arg residue, indispensable for sugar binding, located at the C-terminal carbohydrate recognition domain (CRD), and its corresponding Ile residue, localized to the N-terminal CRD, are shown in bold. Amino acid sequences of different galectins are presented for comparison (bottom). These include: human galectin-1 (Galec-1); human galectin-2 (Galec-2); the carbohydrate binding domain (amino acids 128-263) of rat galectin-3 (Galec-3); N-terminal (Galec-4-Nt) and C-terminal (Galec-4-Ct) halves of galectin-4; N-terminal (CE-Nt) and C-terminal (CE-Ct) halves of a 32-kDa β-galactoside-binding protein from *Caenorhabditis elegans*; N-terminal (Galec-8-Nt) and C-terminal (Galec-8-Ct) halves of galectin-8. Residues with shared identity are boxed. Residues with shared similarity are shaded.

Fig. 3 shows Northern blot analysis of RNA from rat tissues probed with galectin-8 cDNA. *Top*, 30 μg of total RNA from the indicated tissues was electrophoresed, blotted, and probed with labeled galectin-8 PCR product as described in "Experimental Procedures". The migration of the 18S and 28S rRNA are marked. *Bottom*, the same blot was stripped and reblotted with cDNA encoding for GAPDH.

Fig. 4 shows immunoprecipitation of *in-vitro* translation product of galectin-8 by lp-lec8 antibodies. Fifty μl of the ^{35}S-labeled galectin-8, expressed as *in-vitro* translation product (see "Experimental Procedures"), were immunoprecipitated by lp-lec8 antibodies as described in Example 2 herein. Five ml of the total ^{35}S-labeled galectin-8 (total), 5 μl of the fraction not precipitated by the antibodies (Sup), and 50 μl of the immunoprecipitated fraction (IP) were subjected to 12% SDS-PAGE and autoradiography.

Fig. 5 shows binding of tag-free recombinant galectin-8 (r-galectin-8) to lactosyl-Sepharose. Tag-free r-galectin-8 was expressed in pLysS as described under "Experimental Procedures". After centrifugation, 30 ml of the soluble bacterial proteins were purified over 5 ml of lactosyl-Sepharose. r-galectin-8 was eluted with 100 mM lactose in buffer-I, and 1 ml fractions were collected. Ten μl of the total and effluent fractions and 50 μl from each elution fraction were resolved by 12% SDS-PAGE,

transferred to nitrocellulose and Western immunoblotted with lp-lec8 antibodies.

Fig. 6 shows binding of rat hepatic galectin-8 to lactosyl-Sepharose. Five g of rat liver were homogenized in buffer-I as described under "Experimental Procedures" and cytosolic extracts (25 ml) were applied over 5 ml of lactosyl-Sepharose. After extensive washing the bound proteins were eluted with 100 mM lactose in buffer-I. One ml fractions (numbered 1-10) were collected and frozen for a period of 16 h at -20°C. Eluted fractions (N° 3-5) were toughed, centrifuged for 15 min at 12000Xg and the pellets were resuspended in 50 ml sample buffer (34). Ten µg protein of total (A) and effluent (B) fractions as well as 50 µl of the supernatant (C) and resuspended pellet (D) of the eluted fractions (N° 3-5) were resolved by 12% SDS-PAGE, transferred to nitrocellulose and Western immunoblotted with lp-lec8 antibodies (top), or subjected to Coomasie staining (bottom).

Fig. 7 shows inhibition by recombinant galectin-8 of serum-induced ^{3}H-thymidine incorporation into DNA. ^{3}H-Thymidine incorporation into DNA was examined as follows: Confluent CHO cells, grown in 24-well trays (Costar), were starved for 48 hours in serum-free medium. Medium containing 10% fetal calf serum was added to the cells in the presence of rgalectin-8 at the indicated concentrations, and the cells were incubated for 14.5 hours at 37°C. The medium was then washed, and the cells were incubated for 2 hours at 37°C in 1 ml of serum-free medium containing 1% BSA, 20mM Hepes (pH 7.5) and 0.5 µCi/ml [^{3}H]-thymidine. At the end of incubation, the solution was removed, cells were washed 3 times in ice-cold PBS and incubated for 30 min at 4°C in 0.5 ml ice-cold 7.5% trichloroacetic acid. The pellets were washed twice with 98% ice-cold ethanol, dissolved in 0.6 ml of 0.1 M NaOH, and counted using scintillation cocktail containing xylene and Lumax (6:4 ratio, respectively).

Fig. 8 shows chromatography of galectin-8 over a FPLC column. Approximately 100 µg protein was loaded onto Superdex-200 HR (Pharmacia) FPLC column equilibrated with buffer A (PBS, 4 mM β-mercaptoethanol, 2mM EDTA), and run for 60 min at 0.5 ml/min.

O.D. was measured at 215 nm, and the column profile was obtained by running separately standard marks.

DETAILED DESCRIPTION OF THE INVENTION

Galectin-8 is a novel, widely expressed protein of 35 kDa which has the characteristic properties of galectins (S-type mammalian lectins). Three lines of evidence demonstrate that galectin-8 is indeed a novel galectin: i. its deduced amino acid sequence contains two domains with conserved motifs that are implicated in the carbohydrate binding of galectins; ii. *in vitro* translation products of galectin-8 cDNA or bacterially-expressed recombinant galectin-8 are biologically active and possess sugar-binding and hemagglutination activity; iii. a protein of the expected size (34 kDa), that binds to lactosyl-Sepharose and reacts with galectin-8-specific antibodies is present in rat liver and comprises ~0.025% of the total Triton-soluble hepatic proteins.

Overall, galectin-8 is structurally related (34% identity) to galectin-4, a soluble rat galectin with two carbohydrate-binding domains in the same polypeptide chain, joined by a link peptide. Nonetheless, several important features distinguish these two galectins: i. Northern blot analysis revealed that unlike galectin-4 that is confined to the intestine and stomach, galectin-8 is expressed in liver, kidney, cardiac muscle, lung, and brain; ii. unlike galectin-4, but similar to galectins-1 and -2, galectin-8 contains 4 Cys residues; iii. the link peptide of galectin-8 is unique and bears no similarity to any known protein; iv. the N-terminal carbohydrate-binding region (CRD) of galectin-8 contains a unique WG-E-I motif instead of the consensus WG-E-R/K motif implicated as playing an essential role in sugar-binding of all galectins. Together with galectin-4, galectin-8 therefore represents a subfamily of galectins consisting of a tandem repeat of structurally different CRDs within a single polypeptide chain.

As used herein, the term "galectin-8-like protein" refers to a protein derived from any mammal, including humans, which protein presents homology to galectin-8 as defined in the present

invention and has the biological properties of galectin-8.

Galectin-8 was cloned when a λ-ZAP rat liver cDNA library was screened with affinity-purified antibodies directed against a 14-amino acid peptide located at the C-terminal end of the insulin-receptor substrate 1 (IRS-1) (14). Since galectin-8 bears no sequence similarity either to IRS-1, or to the peptide used as immunogen, it was suspected that the reactivity towards IRS-1 antibodies could be due to a false positive reaction. This conclusion is supported by the fact that the anti-peptide antibodies used for screening, failed to react with purified recombinant galectin-8 either by means of immunoprecipitation, or immunoblotting.

The primary structure of galectin-8 resembles that of galectin-4, namely, two homologous (38% identity) carbohydrate-binding regions (CRDs) linked by a short ≈30-amino acids linking peptide. This unique architecture is shared so far only by two galectins: rat galectin-4 (12) and its *C. elegans* homologue (13). Other galectin types, that contain a single CRD, exist and function as non-covalent dimers, which provides them with the potential to aggregate or agglutinate glycoconjugates. Since galectin-4 exists as a monomer, experiments were carried out to determine whether galectin-8 exists as a monomer or a dimer. Separation of galectin-8 over Superdex-200 HR (Pharmacia) FPLC column according to the present invention revealed that galectin-8 exists as a monomer (Fig.8). Hepatic galectin-8 (Fig. 6) has a similar mobility on SDS-PAGE as its recombinant counterpart (Fig. 5). This suggests, though not proves, that hepatic galectin-8 is neither heavily glycosylated, nor it is subjected to extensive post-translational modifications (e. g. phosphorylation).

Although galectin-8 contains two putative CRDs, potential differences in sugar-binding between the domains is predicted from a critical difference in their sequence [WG-E-I vs. WG-E-R at the N- and C-terminal CRDs of galectin-8, respectively (cf. Fig. 2)]. The (bold) Arg residue has been implicated as playing an important role in the interactions between galectins and the glucose moiety of lactose (5). Furthermore, site-directed mutagenesis studies (4) indicate that this conserved Arg is

indispensable for sugar binding. The presence of Ile^{90} (instead of an Arg) at the N-terminal CRD of galectin-8 suggests that this domain might have a different sugar-binding specificity. In that respect galectin-8 resembles galectin-4 whose CRDs are distinct both in structure and sugar-binding specificity (12). The presence of two CRDs with a potential different sugar-binding specificity might be required to achieve high affinity binding to multivalent glycoprotein ligands possessing different sugar moieties.

Like other galectins, galectin-8 lacks a classical signal sequence or a transmembrane segment. Indeed, galectin-8 was isolated from the cytosolic fraction of rat liver. These findings do not exclude the possibility that galectin-8, like other galectins, could be externalized by an atypical secretory mechanism (15). Immunohistochemical studies revealed that secreted galectins are concentrated in evaginations of the plasma membrane, which pinch off to form labile lectin-rich extracellular vesicles which may interact with cell surface proteins (15).Expression of galectin-8 seems to be developmentally regulated. Very low levels of expression were noted in whole embryos, while high levels of expression were noted in adult tissues. In that respect galectin-8 might resemble other galectins that were implicated as regulators of cell growth and embryogenesis (8-10).

The invention will now be described by way of the following non-limiting examples and the accompanying drawings.

EXAMPLES

Experimental Procedures

(a) Materials - Restriction enzymes were purchased from Fermentas. Radiolabeled nucleotides and [^{35}S]methionine were from Amersham (Amersham, Buckinghamshire, UK). All other reagents were from Sigma unless stated otherwise.

(b) Antibodies - Antisera to insulin receptor substrate 1 (anti-IRS-1) were raised in rabbits according to standard procedures, by injection of a peptide CYASINFQKQPEDRQ corresponding to the carboxy-terminal 14 amino acids of rat liver IRS-1 (and an

additional Cys residue at the N-terminal site). Antibodies were affinity-purified from the serum by adsorption onto a column of peptide coupled to Affi-gel 10, elution with 100 mM HCl glycine pH 2.7, and immediate neutralization. Anti glutathione-S-transferase (GST) antisera was a kind gift from Y. Yarden (Weizmann Institute).

(c) Screening of Rat Liver cDNA Expression Library - λ-Zap rat liver cDNA library in the Lambda ZAP II Vector (Stratagene, La Jolla, CA), was screened separately and in duplicate with affinity-purified anti IRS-1 antibodies (see (b) above). Screening was carried out according to the instruction manual provided by the manufacturer (picoBlue™ Immunoscreening Kit, Stratagene, La Jolla, CA). Positive plaques were isolated by three repetitive cycles of the procedure. The ExAssist/SOLR system (Stratagene, La Jolla, CA) was used to allow efficient excision of the Bluescript phagemid from the λ-ZAP vector, and SOLR cells containing positive clones were isolated. Initial DNA sequencing of one positive clone was carried on both strands, using T3 and T7 universal primers with Sequenase version 2.0, (United States Biochemicals, Cleveland, Ohio). Subsequent sequencing was carried out with internal primers designed as the sequencing progressed. All other manipulations of nucleic acids such as restriction, ligations, transformation, gel electrophoresis, blotting, gel elution, radiolabeling, and preparation of buffers were done using standard protocols (16). Search of the GenBank revealed that the isolated clone is unique and it bears no sequence similarity with IRS-1, or the peptide, against which the antibodies were raised. The reason why this clone was picked up by the antibodies remains unclear.

(d) Northern Blot Analysis - RNA extraction was carried out as described (16). Total RNA (30 µg) was electrophoresed, the gel was blotted onto nitrocellulose, and the blot was probed with labeled PCR product which was obtained by the following procedure. Two primers, 5'-CCCGACAATCCCCTATGTCAGTACC-3 and 5'-GCATGGCCAGGCCTGACAACA-3', were used to amplify the entire cDNA coding sequence of galectin-8, using the cloned cDNA in Bluescript as a template. The PCR products were labeled with [α-

^{32}P]-ATP by random priming with DECAprime II DNA labeling kit (Ambion, Austin, TX). Hybridization was carried out at 42 °C in 50% formamide 5 X SSC, and washes were at 60°C in 0.1X SSC, 0.1% SDS.

(e) Expression of recombinant galectin-8 in Escherichia Coli - Expression of galectin-8 as a GST fusion protein (GST-galectin-8) was carried out by using two primers: T7 and 5'-GGGGGGGGATCCATGTTGTCCTTAAGCAAT-3'(the EcoR I, Nde I, and BamH I restriction sites, respectively, in the primer are underlined) to amplify the entire cDNA insert of galectin-8, using the cloned cDNA in Bluescript as a template. The PCR products were digested with BamH I and EcoR I, gel-purified, and ligated into pGeX-2X expression plasmid (Pharmacia) in the TOP_{10} bacterial host (Invitrogen). For direct expression of (tag-free) r-galectin-8, a sense primer 5'-GGGGGGCATATGTTGTCCTTAAGCAAT-3' and an antisense primer 5'-GGGGGGGGATCCGCCATTTTGTATTTCCAG-3' were used to amplify the entire coding sequence of galectin-8, using the cloned cDNA in Bluescript as a template. The PCR products were digested by Nde I and BamH I, gel-purified, and ligated into a pET-3a expression plasmid (Novagen) in the pLysS bacterial host. Sequencing of both expression plasmids was carried out to ensure proper, in-frame, ligation of the inserts.

To express GST-galectin-8, bacteria were cultured in 0.5 liter of LB medium until the absorbance at 600 nm was 0.5. Expression of GST-galectin-8 was then induced with 5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 4 h. To isolate the recombinant protein, a bacterial pellet was isolated by centrifugation, resuspended in 30 ml of buffer I (phosphate buffered saline containing 4 mM β-mercaptoethanol, 2 mM EDTA, 10 µg/ml soybean trypsin inhibitor, 2 mM benzamidine and 1 mM phenylmethylsulfonyl fluoride, pH 7.5), and lysed by sonication. Debris were removed by centrifugation at 38,000 xg at 4 °C for 45 min., and 30 ml of the soluble extract were passed over 5 ml of lactosyl-Sepharose. Unbound proteins were eluted with buffer I, while the lectin was subsequently eluted with buffer I containing 100 mM lactose. A similar procedure was utilized to express r-galectin-8 in the pET-3a expression plasmid, save for the fact

that the bacteria were centrifuged when the absorbance at 600 nm was 0.3, without addition of IPTG. Recombinant galectin-8 was isolated under reducing conditions, since in their absence the protein underwent denaturation even when maintained at 4°C.

***(f)In-Vitro* translation of galectin-8** - For *in vitro* translation of galectin-8, the BamH I/EcoR I-digested PCR product, described above, was cloned into pcDNA I mammalian expression plasmid (Invitrogen). *In vitro* translation in the presence of [^{35}S]-methionine was performed using the TNT *in vitro* translation kit (Promega) according to the manufacturer's instructions.

(g) Immunoprecipitation - lp-lec8 antibodies were added to 60 µl of 50% protein A-Sepharose in 0.1 M Tris buffer, pH 8.5, and were incubated for 1 hr at 4°C. Bacterial cell extracts were prepared in buffer I. 500 µl extracts (0.8 mg) were incubated for 2 hr with the antibody-protein A-Sepharose complex. Immunocomplexes were washed, suspended in sample buffer, resolved on 10-12% SDS-PAGE and transferred to nitrocellulose for Western blotting.

(h) Protein electrophoresis and blotting - Immunoblotting was carried out by standard procedures. The blotted proteins were incubated with lp-lec8 antibodies at 4°C for 16 h and then were extensively washed. To detect antibody binding, a horseradish peroxidase-conjugated Protein A ECL kit (Amersham) was used according to the manufacturer's instructions.

(i) Purification of galectin-8 from rat liver - Freshly isolated rat livers from male Wistar rats were homogenized in buffer I (1 g /5 ml) supplemented with 10 µg/ml aprotinin and 5 µg/ml leupeptin. The homogenate was centrifuged for 1 h at 4°C at 100,000x*g*, and 25 ml of the supernatant were passed over 5 ml of lactosyl-Sepharose, following the procedure described above. The eluted fractions were kept frozen at -20°C. Since intact galectin-8 denatures upon freezing, the frozen fractions were toughed, and centrifuged at 12, 000 xg, for 15 min. at 4°C to precipitate, and thus concentrate, galectin-8. Supernatants and pellets were resuspended in sample buffer, resolved by 12% SDS-PAGE, transferred to nitrocellulose and Western immunoblotted with lp-lec8 antibodies. The amount of galectin-8 in rat liver was estimated using 100,000xg supernatants that were prepared in

buffer I in the presence of 1% Triton-X-100.

(j) **Assay of lectin activity-** The biological activity of galectin-8 was assayed by measuring its ability to agglutinate formaldehyde-fixed, trypsin-treated rabbit erythrocyte. Rabbit erythrocytes were trypsin-treated according to Lis and Sharon (17). Cells were incubated for 1 h at 37°C with 0.1% trypsin in PBS, washed five times in 10 volumes of 0.9% NaCl/packed ml of cells, and resuspended in 0.9% NaCl to yield an erythrocyte suspension with an absorbance of 1.5 at 620 nm. Half ml aliquots of erythrocyte suspension was incubated for 45 in at room temperature with the lectin solution. Aliquots (0.2 ml) of the upper part of the tube were removed, mixed with 0.8 ml of PBS, and the optical density at 620 nm was monitored.

EXAMPLE 1

Isolation of galectin-8, a novel mammalian galectin

A cDNA encoding for a new galectin, termed galectin-8, was cloned from a λ2-Zap rat liver cDNA library (Fig. 1). The isolated clone contained an open reading frame (ORF) (nucleotides 112-1068) with a potential initiation ATG codon at position 121. This ORF coded for 316 amino acids, which form a protein of about 35 kDa. The putative coding sequence was followed by a signal for translation termination (TAG) and 176 nucleotides of 3'-untranslated region. Search of the GenBank for similar nucleotide sequences revealed that this sequence is unique. This sequence, depicted in Fig. 1, has been submitted to the Gen Bank™/EMBL Data Bank with accession number U09824.

Analysis of galectin-8 using alignment algorithms suggested the presence of two homologous domains ≈140 amino acids each, linked by a link peptide of 32 amino acid residues (Fig. 2, top). Thirty eight percent of the amino acids were identical between the first and second domains (Fig. 2, bottom). Both domains contained sequence motifs (e. g. H-NPR; WG-EE) that have been conserved among most carbohydrate recognition domains (CRDs) of galectins analyzed so far. Structurally, galectin-8 resembles a 32-kDa β-galactoside-binding protein from *Caenorhabditis elegans* (13) (CE-galectin), and rat galectin-4 (galectin-4) (12), that

also contain two CRDs connected by a link peptide (Fig. 2). At the level of nucleic acids, galectin-8 is 50% and 45% homologous to galectin-4 and CE-galectin, respectively. At the level of amino acids, galectin-8 shares 34% and 31% identity, respectively, with the above proteins. No homology with any known protein was found in the region of the link peptide. Like other galectins, galectin-8 lacks classical signal sequence or transmembrane segment, but it contains three potential N-linked glycosylation (Asn-X-Ser/Thr) sites. Analysis of its predicted secondary structure (not shown), revealed that the N-and C-terminal domains of galectin-8 share a great degree of structural homology, as expected from their primary structure. Both domains are predicted to form several β-sheets, a structural feature of other galectins (5).

The cDNA clone encoding galectin-8 may be used as a probe to isolate and characterize the full length genomic sequence encoding this protein in various mammals, for example, humans and rats, using standard procedures.

Further, the above mentioned cDNA clone and/or the full-length genomic sequence encoding galectin-8 may be used to generate, by standard procedures, fragments containing only a portion of the full-length galectin-8 sequence, where each fragment essentially retains at least one of the biological activities of galectin-8. These fragments are termed 'biologically active fragments'. Moreover the galectin-8 sequence may also be used to generate analogs of galectin-8 (herein termed "galectin-8-like proteins") or fragments thereof, such analogs having at least one amino acid residue added, deleted or replaced by another in comparison to the native galectin-8 sequence, and such analogs essentially retaining the biological activity of their non-modified progenitor molecules.

EXAMPLE 2

Antibodies against the link peptide of galectin-8 (lp-lec8) or against recombinant galectin-8 (rgalectin-8)

Since galectin-8 contains a unique link peptide region, antibodies against this region are not expected to cross-react

with other galectins. A peptide corresponding to positions 168-182 in the link peptide of galectin-8 (and an additional Cys residue at the N-terminal site) of the sequence CQISKETIQKSGKLHL was synthesized, purified, and polyclonal antibodies against it were raised in rabbits by standard procedures. The antibodies (denoted lp-lec8) were affinity-purified over a column of immobilized peptide. lp-lec8 antibodies reacted specifically with galectin-8 both by means of immunoprecipitation (IP) and immunoblotting (IB). Furthermore, these interactions could be specifically blocked in the presence of 1μM peptide (not shown). Since lp-lec8 antibodies specifically react with the link peptide of galectin-8, antibodies towards whole recombinant galectin-8 were generated as well. Purified tag-free rgalectin-8 was used as immunogen for injection into rabbits, and antibodies were affinity purified over columns of Protein A coupled to agarose. These antibodies reacted specifically with galectin-8 both by means of immunoprecipitation and immunoblotting.

These antibodies are most useful for identification of naturally occurring degradation products of galectin-8, where the link peptide region has been deleted, or proteins homologous to galectin-8 in domains different from the link peptide region". Cross-reactivity with homologous proteins is assessed by the ability of lp-lec8 antibodies to react with the suspected candidates, and by the ability of peptides, directed against unique regions of galectin-8, outside the link peptide region, to compete with galectin-8 antibody binding.

EXAMPLE 3

In-vitro-translated galectin-8 is biologically active.

Galectin-8 cDNA was transcribed and translated *in vitro* using a TNT (Promega) kit. An ^{35}S-labeled product of the expected size (34 kDa) was synthesized (Fig. 4). This *in vitro*-translated product was indeed galectin-8 since it could be immunoprecipitated with lp-lec8 antibodies described in Example 2 (Fig. 4). As predicted by its primary amino acid sequence, *in vitro*-translated galectin-8 exhibited the key feature of galectins, namely, capacity to bind to a column of lactosyl-

Sepharose in the presence of reducing agents, and to be eluted with 0.1 M lactose (not shown).

EXAMPLE 4

Recombinant galectin-8, expressed in bacteria, remains soluble and retains lectin biological activity

To further characterize galectin-8, it was expressed in bacteria as a GST-fusion protein. GST-galectin-8 remained bound to glutathione-Sepharose beads, and could be eluted with glutathione (not shown). GST-galectin-8 retained its sugar-binding capacity and could be purified by binding to lactosyl-Sepharose and elution with 0.1 M lactose (not shown). Routinely, 3 mg GST-galectin-8 could be purified in such a way from 1 liter of bacterial extracts. Like other galectins, GST-galectin-8 also maintained hemagglutination activity. Half and maximal activities were obtained with 0.1 and 1 µg/ml of GST-galectin-8, respectively.

In a different approach a tag-free rgalectin-8 was expressed employing a pET-3a expression plasmid (Novagen) in the pLysS bacterial host. Unlike intestinal recombinant galectin-4 that precipitates and cannot be extracted with buffers that preserve its lectin activity (12), rgalectin-8 could be readily extracted from bacteria in a soluble form. rgalectin-8 was not subjected to major proteolytic cleavage, as it migrated at the expected size of 34 kDa. Most important, rgalectin-8 retained its sugar-binding activity and 1.2 mg protein/liter bacteria were obtained following its purification over lactosyl-Sepharose column (Fig. 5).

To optimize expression, the induction time and the concentration of IPTG is varied. To further purify GST-galectin-8 or rgalectin-8, approximately 5 mg protein are loaded onto a column of antibodies covalently linked to Affi-Gel 15 beads (Pharmacia). The bound proteins are then eluted with HCl/glycine buffer (pH 2.8) and immediatly neutralized.

EXAMPLE 5

Endogenous galectin-8 is present in rat liver

To demonstrate the presence of endogenous galectin-8 in rat liver, a cytosolic (100,000xg supernatant) liver extract was prepared, applied to a column of lactosyl-Sepharose, and proteins retained specifically by the column were eluted with 0.1M lactose. Advantage was taken of the fact that hepatic galectin-8 denatures and precipitates upon freezing. Fractions, eluted from the lactosyl-Sepharose column, were therefore frozen at -20°C, thawed, and centrifuged to precipitate, and thus concentrate, the hepatic galectin-8. Staining with Coomasie Blue revealed that most hepatic proteins failed to interact with lactosyl-Sepharose and therefore remained in the flow-through fraction (Fig. 6, bottom). Immunoblotting with lp-lec8 antibodies (Fig. 6, top) revealed that while hepatic galectin-8 could not be detected in total cytosolic liver extracts, a 36 kDa protein, with the expected size of galectin-8, remained bound to, and could be eluted from the lactosyl-Sepharose column. Hepatic galectin-8 was readily detected in the pellets, but not in the supernatants of the (frozen and thawed) eluted fractions, indicating that indeed it denatures upon freezing. These results suggest that functionally active cytosolic galectin-8 is present in rat liver (Fig. 6).

To estimate the amounts of galectin-8 in rat liver, Triton-soluble liver extracts were prepared, and resolved by means of SDS-PAGE. Known amounts of rgalectin-8 were run in parallel. All samples were then subjected to Western immunoblotting, using anti-rgalectin-8 antibodies. Assuming that the immunoreactivity of rgalectin-8 and the endogenous hepatic protein are comparable, we calculated that ~25 ng of galectin-8 are present in 100 mg of Triton-soluble liver extracts. These findings suggest that galectin-8 comprises ~0.025% of total Triton-soluble hepatic proteins.

EXAMPLE 6

Galectin-8 is widely expressed. Tissue distribution and cellular localization of galectin-8.

Identifying tissues where galectin-8 is highly expressed provides important clues related to its possible function and

involvement in development. More important, determining whether galectin-8, like other galectins, is externalized, is of fundamental importance in attempts to assess its mode of action. Three different approaches may be used to gain a detailed tissue distribution of galectin-8. i. Northern blot analysis of rat tissues; ii. to ascertain that the level of mRNA indeed reflects the level of expression of galectin-8, the abundance of galectin-8 in various tissues may be determined by Western blot analysis using anti-rgalectin-8 antibodies. Since galectin-8, like other galectins, is prone to proteolysis, freshly isolated tissues are directly homogenized in 4M guanidinium-HCl to inactivate all proteases. The amount of galectin-8 in the tissue under study is determined following SDS-PAGE, Western blotting, and probing with anti-rgalectin-8 antibodies. iii. In addition, tissues of interest (e. g. liver and brain) will be studied in more detail by *in-situ* hybridization. In preliminary studies, *in situ* hybridization of brain slices indicated that galectin-8 is specifically expressed in the hypocampus, cerebellum, and brain stem, with little expression in the cortex (not shown). These findings suggest that unlike galectin-4, galectin-8 is an abundant protein that might play a role in certain brain functions.

Northern blot analysis of rat tissues was carried out and the results are shown in Table 1.

Table I. Tissue Distribution of galectin-8 mRNA according to Northern Blot Analysis.

Lung	100
Liver	43.4
Cardiac muscle	39.5
Spleen	36.3
Hind limb Muscle	31.6
Brain	12.6
Fetus	8.1

Total RNA from the indicated rat tissues was electrophoresed, blotted, and probed as described in legend to Fig. 3. The intensity of the signal corresponding to the galectin-8 probe was determined by densitometry and is presented as percentage of the strongest signal (normalized to GAPDH) which was obtained in lung (100%).

The expression of galectin-8 in different rat tissues was examined by Northern blots (Fig. 3). A single mRNA transcript of ~ 3 kb hybridized with galectin-8 PCR product probe. Unlike galectin-4, which is confined to intestine and stomach (12), galectin-8 mRNA is highly expressed in lung, and to a lower extent in liver, kidney, spleen, hind-limb, and cardiac muscle (Fig. 3, Table 1). Lower levels of expression were detected in brain and almost no expression was found in whole rat embryos.

EXAMPLE 7

Generation and purification of recombinant N-terminal (rgalectin-8nt) and C-terminal (rgalectin-8ct) domains of galectin-8.

To determine whether galectin-8nt has any sugar-binding activity, and whether galectin-8ct might function independently of its N-terminal half, galectin-8nt and galectin-8ct are amplified by PCR and proper restriction sites are introduced. Expression of each domain either as a GST-fusion protein or as tag-free domain are carried out as described above (Example 4). To express tag-free galectin-8ct the Met residue placed within the MCS of pET-3d is utilized as the start-site. Purification of galectin-8nt and galectin-8ct is carried out as described above (Example 4).

EXAMPLE 8

Generation of mammalian cells that overexpress galectin-8 in a transient or stable manner.

The cDNA coding for galectin-8 was introduced into four different eukaryotic high expression plasmids: pcDNA I Amp (Invitrogene); pREP8 (Invitrogene); pBPV-II, and pMAMneo (Clontec). The latter plasmid, having a dexamethasone-inducible MMTV-LTR promoter is of particular use if constitutive

overexpression of galectin-8 induces growth arrest or prevents adhesion of the transfected cells. Sequencing of the vector/insert boundaries is carried out, to ensure proper integration of the insert.

a. Transient expression of galectin-8- Northern blot analysis of RNA and Western immunoblotting with lp-lec8 antibodies, has indicated that COS-7 cells express low levels of endogenous galectin-8. These cells are therefore appropriate targets to study transient expression of galectin-8. COS-7 cells are plated in DMEM/10% FCS at 2x 10^6 cells /10 cm plate, 24 h before transfection. Cells are transfected with 10 µg of plasmid DNA using DEAE-dextran and DMSO-facilitated uptake according to standard procedures (modified by 0.1 mM chloriquine treatment). Cells are harvested 48-72 h thereafter, and the expressed galectin-8 is detected by Western immunoblotting with lp-lec8 or rgalectin-8 antibodies. Galectin-8 is purified by affinity-chromatography over lactosyl-Sepharose column, and by immunoaffinity chromatography using lp-lec8 or rgalectin-8 antibodies coupled to Sepharose as immunoadsorbent.

b. Stable expression of galectin-8- The above expression plasmids are used for stable transfection of galectin-8 DNA into Chinese Hamster Ovary (CHO) cells that have relatively low amount of endogenous galectin-8. Stable transfectants are identified by their ability to accumulate galectin-8 in the cytosol, or to secrete galectin-8 into the medium. Conditioned-medium is collected, concentrated by Amicon Centricon-10 micro concentrator, and lyophilized. Cytosolic extracts are prepared by boiling in "sample buffer" and the presence of galectin-8 is detected by immunoblotting with galectin-8 antibodies. Cells expressing the highest concentration of galectin-8 are further propagated.

EXAMPLE 9

Biological activity of whole rgalectin-8 and its individually-expressed N- or C-terminal domains

To assess the functional need for two CRDs within the same polypeptide chain of galectin-8, the biological activity of

rgalectin-8 is compared with that of its individually-expressed domains.

i. Hemagglutination activity of rgalectin-8, rgalectin-8nt and rgalectin-8ct is assayed as previously described (17). Rabbit erythrocytes are trypsin-treated and fixed with glutaraldehyde. Following washings in 0.1 M glycine/PBS and PBS, and proper dilution, hemagglutination activity of serial dilutions of rgalectin-8 is compared with those of rgalectin-8nt and rgalectin-8ct. If rgalectin-8, like galectin-1, is capable of forming homodimers, and if both CRDs of galectin-8 are capable of sugar binding, then rgalectin-8 is expected to express hemagglutination activity. If however rgalectin-8nt has reduced or no sugar-binding activity, and if rgalectin-8 fails to dimerize, then rgalectin-8, having a single functional CRD at the C-terminal domain, might fail to express hemagglutination activity. These results will implicate galectin-8 as having a function different from cross-linking glycoconjugates.

ii. Carbohydrate-binding specificity of whole galectin-8 and its individually-expressed domains is compared to previously determined specificity of other galectins, including galectin-4. To avoid possible alterations in the native structure of galectin-8 (e.g. due to carboxymethylation and iodination) 5 µg of purified rgalectin-8 (or individual domains) are incubated with 100 µl of lactosyl-Sepharose; conditions that result in quantitative binding of rgalectin-8. Binding specificity may be determined by the capacity of various saccharides (e.g. thiodigalactose, thiodiglucose) to inhibit binding of rgalectin-8 (or individual domains), when compared with lactose. If galectin-8nt expresses, as predicted, altered or markedly reduced carbohydrate-binding activity, binding activity may be restored by site-directed mutagenesis, where the Ile-90 residue is mutated to Arg.

EXAMPLE 10

Site-directed mutagenesis.

Site-directed mutagenesis is carried out using "Altered Sites II in vitro mutagenesis systems" (Promega) according to the

manufacturer's manual. First, Ile-90 is mutated to Arg to determine how such substitution affects hemagglutination activity and sugar binding specificity of rgalectin-8nt and whole galectin-8. Conversely, Arg-253, located within the WG-E-R motif at the C-terminal CRD may be mutated to Ile, and the effect of this mutation on the biological activity of galectin-8 is assessed. If Arg-253->Ile mutation markedly reduces or abolishes the *in vitro* biological activity of galectin-8, then the biological consequences of overexpression of this negative-dominant mutant will be compared with cells that overexpress the native form of galectin-8.

EXAMPLE 11

Sensitivity of rgalectin-8 to oxidation.

One whole mark of certain galectins is the sensitivity of their carbohydrate-binding activity to oxidation. Other studies suggest that for certain of these lectins the thiol-dependence may be ascribed to an artifact of the extraction procedure rather than an intrinsic requirement of the protein itself. To assess whether galectin-8 requires reducing environment to remain biologically active, the effects of various reductants and oxidants on the binding activity of galectin-8 to lactosyl-Sepharose are studied as described for other galectins. If galectin-8 activity is sensitive to oxidation, alkylation of rgalectin-8 may be carried out with iodoacetamide or with N-ethyl-maleimide. The modified product is then subjected to rechromatography over lactosyl-Sepharose column and is eluted with water. Alkylation, that stabilizes galectin-1, may preserve and stabilize rgalectin-8 activity (i.e. binding affinity to lactosyl-Sepharose), and enables increase of the half-life of rgalectin-8 and better study of its effects on cultured cells under the oxidizing environment of tissue culture medium.

EXAMPLE 12

Sensitivity of rgalectin-8 to proteolysis.

Preliminary experiments have indicated that endogenous mammalian galectin-8 is susceptible to proteolysis. To determine

the physiological significance of this phenomena, pulse-chase experiments with ^{35}S-labeled cells, followed by immunoprecipitation of the endogenous galectin-8, are carried out in CHO cells overexpressing galectin-8. ^{35}S-labeled galectin-8 is precipitated with lp-lec8 or rgalectin-8 antibodies. The half-life of endogenous galectin-8 and the formation of *in vivo* degradation products are then evaluated. To distinguish proteolysis that occurs *in vivo* from one that occurs during extraction and purification, homogenization is carried out in the presence of trace amounts of ^{125}I-labeled rgalectin-8.

EXAMPLE 13

Biological activity of galectin-8

The effects of galectin-8 on cell adhesion and on regulation of cellular growth are examined.

Effects of galectin-8 on cell adhesion

One of the well characterized effects of galectin-1 is its ability to inhibit myoblast adhesion to laminin (15). To determine whether galectin-8 shares a similar property, the effects of overexpression of galectin-8 on cell adhesion are studied. COS-7 cells are co-transfected with an expression vector for β-galactosidase (pSMβGal) at a 1:20 ratio to the galectin-8 vector. Cells expressing β-galactosidase are easily distinguished by a blue staining after histochemical reaction with X-gal, 36 h following transfection. Alterations in adhesion of blue cells as a function of time are monitored. Control cells are cotransfected with pSMβGal and pcDNA-IR (which contains an insert encoding for the insulin receptor). If positive results are obtained, thio-D-glucose (TDG) is added to inhibit lectin-carbohydrate interactions and study the contribution of the carbohydrate-binding domains to this effect.

In an alternative approach CHO cells, transfected with the pMAMneo-galectin-8 plasmid (which has a dexamethasone-inducible MMTV-LTR promoter) is used. Their adhesive properties to the culture dish, before and after induction, are compared. If positive results are obtained, the effects of TDG on cell adhesion and the effects of exogenously-added rgalectin-8 on non-

induced cells are determined.

Function of galectin-8 as a cytostatic factor and cell growth regulator.

mGBP, a single-domain homologue of galectin-8, was shown to be a cell growth-regulatory molecule and a cytostatic factor that binds to a specific cell surface receptor (8). To determine whether galectin-8 fulfills a similar role, since galectin-8 is expressed in rat liver, rat hepatoma (Fao) cells are used as a model system. Another model is mouse embryo fibroblasts (MEF), that were already shown to be subjected to the growth inhibitory action of mGBP (8). Growth inhibition induced by purified rgalectin-8 is assessed by several parameters:

i. Direct counting of logarithmically growing cells, incubated for increasing time periods with increasing concentrations of native or denatured (control) rgalectin-8. Cell viability is assessed colorimetrically utilizing the neutral red uptake assay.

ii. Inhibition of DNA synthesis is monitored by [^{3}H] thymidine incorporation into control, and rgalectin-8-treated cells.

iii. Change in population distribution, due to inhibition of cell growth, is assessed by FACS analysis.

iv. Changes in cell morphology are monitored in cells grown on cover slips. Following treatment, cells are washed, fixed, and viewed by Nomarski interference contrast microscopy.

The reversibility of the galectin-8 effects on these parameters may then be evaluated. The relation between sugar binding and the biological activity of rgalectin-8 may be further assessed by the ability of 10 mM TDG to compete for rgalectin-8 binding. Successful results lead to the second stage of the study, where it is determined whether growth inhibition is related to the growth state, as is in the case of mGBP and cytokines. For that purpose cells stationed in Go by serum starvation, and cells rescued from Go by serum stimulation, are treated with galectin-8 for different times, and its potency to attenuate or inhibit cell growth is evaluated.

Inhibition of DNA synthesis was monitored in control and rgalectin-8-treated CHO cells as described in the legend to Fig. 7. It can be seen that rgalectin-8 inhibits serum-induced [^{3}H]

thymidine incorporation in a dose-dependent manner. Half-maximal effects are obtained at 0.5μM and maximal effects at 2μM rgalectin-8, GST alone is without effect.

EXAMPLE 14

Use of galectin-8 antibodies as diagnostic tools for neoplastic transformation.

Suitable compositions prepared by well-known standard procedures, containing anti-galectin-8 antibodies may be used to detect overexpression of this protein following neoplastic transformation in general, and in metastatic melanoma cells in particular, and accordingly, to determine whether overexpression of galectin-8 can serve as an early signal for neoplastic transformation, and/or the development of metastatic melanoma. Thus, the anti-galectin-8 antibodies mat serve as a diagnostic tool for early detection of the above disease. Moreover, the presence of a subject's own anti-galectin-8 antibodies can also serve as such a diagnostic tool, which endogenous anti-galectin-8 antibodies may be assayed with purified galectin-8.

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CLAIMS

1. Galectin-8, a galectin-8-like protein and fragments thereof selected from the group consisting of:

(i) the protein galectin-8 of the amino acid sequence depicted in Fig. 1;

(ii) a protein having greater than about 80 percent similarity to all or part of the sequence of amino acid residues 1-316 depicted in Fig. 1;

(iii) a protein having greater than about 80 percent similarity to all or part of the sequence of amino acid residues 1-151 depicted in Fig. 1;

(iv) a protein having greater than about 80 percent similarity to all or part of the sequence of amino acid residues 152-316 depicted in Fig. 1;

(v) a protein of (i), (ii), (iii) or (iv) in which one or more amino acid residues have been added, deleted, replaced or chemically modified without substantially affecting the biological activity of the protein;

(iv) a biologically active fragment of (i) to (v); and

(vii) an homologous polypeptide to that of (i) to (vi) derived from another mammal and which has a similar biological activity to that of (i) to (iv).

2. Galectin-8 according to claim 1 of molecular weight of about 35 Kd and the amino acid sequence depicted in Fig. 1.

3. An isolated DNA molecule encoding a polypeptide product having all or part of the primary structural conformation of galectin-8 or of galectin-8-like protein and having the biological activity of galectin-8.

4. An isolated DNA molecule according to claim 3 encoding galectin-8, said DNA having the nucleotide sequence depicted in Fig. 1.

5. A DNA molecule according to claim 3 or claim 4 selected from the group consisting of:

(i) a DNA molecule having a nucleotide sequence derived from the coding region of a native galectin-8 gene;

(ii) a DNA molecule capable of hybridization to the cDNA clones of (a) under moderately stringent conditions and which encodes biologically active galectin-8; and

(iii) a DNA molecule which differs, as a result of the degenerative nature of the genetic code, from the DNA sequences defined in (i) or (ii) and which encodes biologically active galectin-8.

6. A DNA molecule according to any one of claims 3 to 5, selected from the group consisting of:

(i) a DNA molecule comprising the coding nucleic acid sequence depicted in Fig. 1;

(ii) a DNA molecule having a nucleic acid sequence of (i) in which one or more codons has been added, replaced or deleted in a manner that the polypeptide encoded by said sequence essentially retains the same biological properties as the polypeptide encoded by an unaltered DNA sequence;

(iii) a DNA molecule encoding a polypeptide encoded having an amino acid sequence of a polypeptide encoded by the DNA molecule of (i) or (ii) but which differs therefrom in view of the degenerative nature of the genetic code;

(iv) a DNA molecule having a coding nucleotide sequence, which is homologous to the DNA molecule of (i), (ii) or (ii), which is derived from a mammal other than rat and which encodes a polypeptide having a similar biological activity to that encoded by the sequence of (i), (ii) or (iii);

(v) a fragment of the coding sequence of (i)-(iv) which encodes a polypeptide which essentially retains the biological properties of the polypeptide encoded by the unfragmented DNA molecule; and

(vi) a DNA molecule comprising the coding DNA sequence of a fragment of (i)-(v) and additional DNA sequence in the 3' and 5' ends.

7. A recombinant DNA molecule comprising a DNA coding

sequence according to any one of claims 3 to 6.

8. A recombinant expression vector comprising a DNA molecule according to any one of claims 3 to 7.

9. A host cell containing a recombinant expression vector according to claim 8.

10. A process for preparing galectin-8 or a galectin-like protein or a biologically active fragment thereof, comprising culturing a suitable host cell according to claim 9 under conditions promoting expression.

11. A pharmaceutical composition comprising as active ingredient an effective amount of galectin-8 or of a galectin-8-like protein according to claim 1 or 2 and a suitable diluent or carrier.

12. A composition according to claim 11 for cell-growth regulation.

13. A composition according to claim 12 for inhibition of cell proliferation.

14. Anti-galectin-8 antibodies.

FIG. 1

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1     AATTCCCCCCCCTGGC TGGGGACAAGTTA TTACT TTGAGTAATCCTTAAA TGAAGAGTGGG 60
61    TAAAGCCCAT ATACGG AAGAGAGACTCCAGTCAACAATATCAA TAAGTTG AAGAAGA AA A 120

121   ATGTTGTCC TTAAGC AATC TACAAAATA TCATCTATAACCCGACAATCCCC TATG TCAG T 180
      Met Leu Ser Leu Ser Asn  Leu Gln Asn  Ile  Ile Tyr Asn  Pro Thr  Ile Pro Tyr  Val  Ser

181   ACCA TTACTGAGCAGTTGAAGCCTGGCTCTTTGATCGTGATCCGTGGCCATGTT CC TAA A 240
      Thr  Ile  Thr Glu  Gln Leu Lys Pro  Gly Ser Leu  Ile  Val  Ile  Arg  Gly  His Val  Pro  Ly

241   GAT TCAGAAAGATTCCAAGTAGACTTTCAGCATGGCAACAGCCTGAAGCCGAGAGCT GAT 300
      Asp  Ser Glu Arg Phe Gln Val Asp Phe Gln His  Gly Asn  Ser Leu Lys  Pro Arg  Ala  Asp

301   GTGGCCTTCCAC TTTAACCCTCGCTTCAAAAGGTCCAACTGC ATTGTTTGTAAC ACACTG 360
      Val  Ala Phe His  Phe Asn Pro Arg Phe Lys Arg Ser Asn Cys  Ile  Val Cys Asn Thr  Leu

361   ACAAATGAGAAATGGGGCTGGGAGGAGATCACCCACGACATGCCTTTCAGAAAAGAAAAG 420
      Thr Asn Glu Lys  Trp  Gly Trp  Glu  Glu  Ile  Thr  His Asp Met Pro Phe Arg Lys Glu  Lys

421   TCCTTTGAG ATTGTGATCATG GTGCTAAAGAACAAA TTCCACGTGGCTGTGAATGGAAAG 480
      Ser Phe Glu  Ile  Val  Ile Met  Val  Leu Lys  Asn Lys Phe His  Val Ala  Val  Asn Gly Lys

481   CACATTCTGCTG TATGCCCACAGGATCAACCCAGAGAAGATAGACACACTGGGCATCTTC 540
      His  Ile Leu  Leu Tyr  Ala  His  Arg  Ile Asn Pro Glu  Lys  Ile Asp Thr Leu  Gly  Ile Ph

541   GGCAAAGTGAACATTCAC TCCATCGGGTTCAGATTCAGCTCGGATTTA CAGAGTATGGAA 600
      Gly  Lys Val Asn  Ile His  Ser  Ile Gly Phe Arg Phe Ser Ser Asp Leu  Gln Ser Met  Glu

601   ACA TCTACTCTGGGACTGACACAG ATAAGTAAAGAAAATATACAAAAGTCTGGCAAGCTC 660
      Thr  Ser Thr Leu  Gly Leu Thr  Gln  Ile  Ser Lys Glu Asn  Ile  Gln Lys Ser  Gly  Lys Leu

661   CAT TTGAGCCTGCCATTTGAAGCAAGGTTGAATGCCTCCATGGGCCCTGGACGAACCGTT 720
      His  Leu Ser Leu  Pro Phe Glu Ala  Arg Leu Asn Ala Ser Met  Gly Pro  Gly  Arg Thr Val

721   GTC GTTAAAGGAGAAGTGAATACA AATGCCACAAGCTTTAATGTTGACCTAGTGGCAGGA 780
      Val  Val Lys  Gly Glu  Val Asn Thr  Asn  Ala Thr  Ser Phe Asn Val Asp Leu Val  Ala  Gly

781   AGGTCAAGGGATATC GCTCTGCACTTGAACCCACGCCTGAATGTGAAAGCGTTTGTAAGA 840
      Arg  Ser Arg Asp  Ile  Ala  Leu  His Leu Asn Pro Arg  Leu Asn Val Lys  Ala Phe Val Arg

841   AACTCC TTTCTTCAGGAT GCCTGGGGAGAAGAGGAGAGAAACATTACCTGCTTCCCATTT 900
      Asn Ser Phe Leu Gln Asp Ala  Trp  Gly  Glu  Glu Glu Arg Asn  Ile  Thr Cys Phe ProPhe

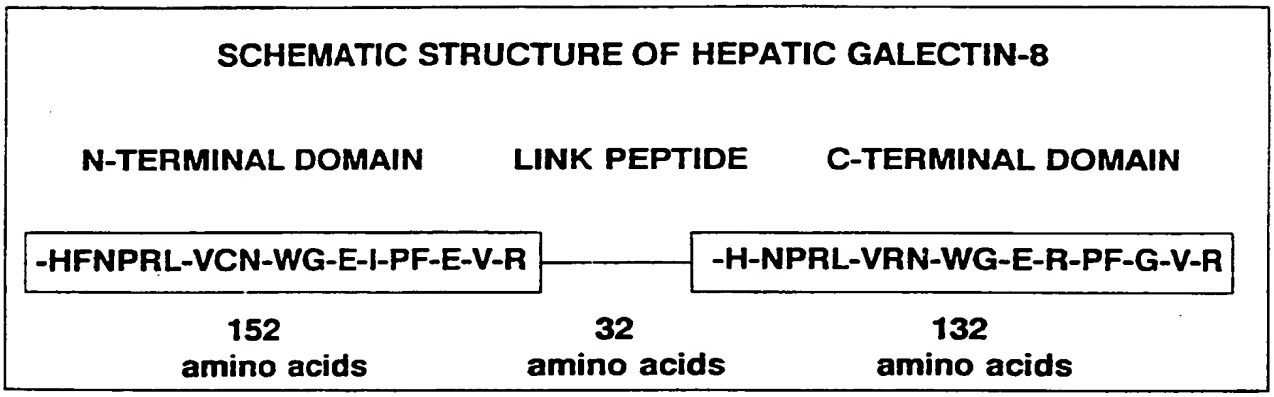
901   AGT TCTGGGATGTACTTT GAGATGATA ATT TACTGTGATGTCCGAGAGTTCAAGGTTGCA 960
      Ser  Ser  Gly Met Tyr Phe  Glu Met  Ile  Ile Tyr Cys Asp Val  Arg  Glu Phe Lys Val Ala

961   GTA AATGGTGTGCACAGCCTGGAGTACAAGCACAGATTTAAAGAC CTAAGCAGCATCGAC 1020
      Val  Asn Gly Val His  Ser Leu  Glu Tyr Lys  His  Arg Ph  Lys Asp Leu S r S r  Ile  Asp

1021  ACACTAGCAGTTGAT GGCGATATCCGTTTGCTGGATGTAAGGAGCTGGTAGCTATCATGA 1080
      Thr  Leu Ala Val  Asp  Gly Asp  Ile Arg Leu  Leu Asp Val  Arg Ser  Trp ***

1081  CTGCCAGAACC CTG GAAATACAAAATGGCTTATCCGATACTGGCCATGTCAAATGCATCT 1140
1141  CGC TTTCACCACAT TGTTATACTGTTAAGTTGAGCTCGCACAACATCAAGTCCTACTGGT 1200
1201  GTT GTCAGGCCTGGCCATGCAGTGTGGCTACCTCTGAATTCCCAGGA                1247
```

FIG. 2A



Galec-1	. . . . .	. . . . . MAGG	LVASN LN LKP	GEC LRVRGEV	24
Galec-2	. . . . .	. . . . . MTGE	LEVKNMD MKP	GS T LKI TGSI	24
Galec-3	. . . . .	. . . . . V PYD	MPLPG . G VMP	RM L I TI I GTV	23
Galec-4-Nt	MAYVPAPGYQ	PT YNPTLPYK	RPI PG . GLSV	GMS IYIQGI A	39
Galec-4-Ct	MNSLPVMAGP	PI FNPPVPYV	GTLQG . GLTA	RRT I I I KGY V	39
CE-Nt	. . .. . . MSAE	EP KSYPVPYR	SVLQE . KLEP	GQT LI VKGS T	33
CE-Ct	. . . . .	. . . . PVPYE	SGLAN . GLPV	GKS L LVFGT V	24
Galec-8-Nt	.M LSLSNLQN	I I YNP TI PYV	STLT E . QLKP	GS LI VI RGHV	38
Galec-8-Ct	. . . . .	. . . . PEE	ARLNA . SMGP	GRTV VVKGEV	22

Galec-1	APDAKSE VLN	LGKD . . . . . S	NNLCEHENPR	ENAHGDAN TI	59
Galec-2	ADGTDGE VIN	LGQG . . . . . T	DKLNEHENPR	ES . . . . ES TI	55
Galec-3	KPNANSE TLN	EKKG	NDI AEHENPR	ENEN . NRR VI	56
Galec-4-Nt	KDNMRREHVN	EAVGQDE . .G	ADI AEHENPR	EDGW. DK . . V	74
Galec-4-Ct	L PTAKN I I I N	EKVG . . . . ST	GDIAEHMNPR	I G . D CV	70
CE-Nt	I DESQRE TI N	LHSKTADFSG	NDVPLHVSVR	EDE . . GK . . I	69
CE-Ct	EKKAKRE HVN	L . . . LRK . . N	GDI SEHENPR	EDEK . H . . . V	55
Galec-8-Nt	PKDSERE QVD	EQHGNSLKPR	ADV AEHENPR	EKRS . N . . CI	75
Galec-8-Ct	NTNATSE NVD	LVAG . . . . RS	RDI A IHI NPR	I NVK . . . . AF	54

FIG. 2B

Galec-1	VCNSKDGGAW	GTEQRE . AV F	PEQPGS VAEV	CIT FDQANET	98
Galec-2	VCNSLDGSNW	GQEQRE . DHL	CESPGS EVKF	TVT FES DKEK	94
Galec-3	VCNTKQDNNW	GREERQS AF .	PEESGK PEKI	QVLVE ADHEK	95
Galec-4-Nt	VFNTMQSGQW	GKEEKKK S.M	PEQKGH HEEL	VEMVMSEHKK	113
Galec-4-Ct	VRNSYMNGSW	GSEERK I PYN	PEGAGQ FEDL	SI RCGTDREK	110
CE-Nt	V LNSFSNGEW	GKEERK . S .N	PI KKG D SEDI	RI RAHDDREQ	107
CE-Ct	VRN SLAANEW	GNEEREGK. N	PEEKG VGEDL	VI QNEEYAEQ	94
Galec-8-Nt	VCN TLTNEKW	GWEEI THD. M	PERKE KEEE I	V IMVLKNKEH	114
Galec-8-Ct	VRNSFLQDAW	GEEERN I TCF	PESSGMYEEM	I I YC DVREEK	94

Galec-1	VK LPDGYEFK	SPNRL . NLEA	I N YMAADGDF	KI KCVAFD . .	135
Galec-2	VK LPDGHELT	SPNRL . GHSH	LSYLSWRGGF	NPSSFKLKE .	132
Galec-3	VA VNDVHLLQ	YNHRMKNLRE	I SQLG I I GDI	TLTS ASHAMI	135
Galec-4-Nt	VV VNGT PFYE	YGHRL . PLQM	VTHLQV DGDL	ELQSI NF LGG	152
Galec-4-Ct	VF ANGQHLFD	RSHRF QAPQR	VDMLEI KGDI	TLS YVQ I . . .	147
CE-Nt	SI VDHKEFKD	YEHRL . P LSS	I S H LSIDGDL	YLNHVHWGGK	146
CE-Ct	VF VNGERYI S	RAHRA . DPHD	I AGLQIS GDI	ELSGI QI Q . .	131
Galec-8-Nt	VA VNGKHI LL	YAHR I NP. EK	I DTLG IF GKV	NI HSI G FRFS	153
Galec-8-Ct	VA VNGVHSLE	YKHRF KD LSS	I DTLAVD GDI	RLLDVRSW . .	132

Galec-1	. . . . .	. . . . .		.	135
Galec-2	. . . . .	. . . . .		.	132
Galec-3	. . . . .	. . . . .		.	135
Galec-4-Nt	QP AASQYPGT	MT I PA YP SAG	YNPPQ	.	177
Galec-4-Ct	. . . . .	. . . . .		.	147
CE-Nt	. . . . .	. . . . .		.	146
CE-Nt	. . . . .	. . . . .		.	131
Galec-8-Nt	SD LQSMETS T	LGL TQ IS KEN	IQKSGK LHLS	L	184
Galec-8-Ct	. . . . .	. . . . .		.	132

FIG. 3

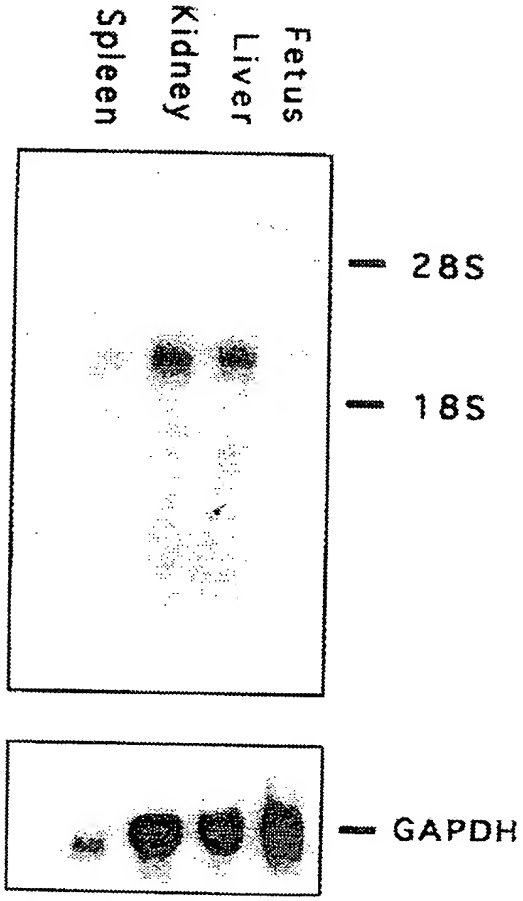


FIG. 4

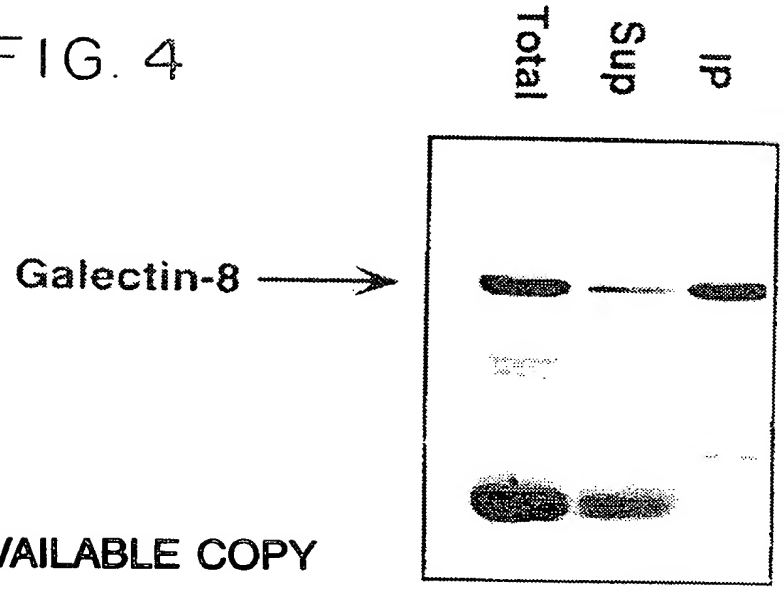


FIG. 5

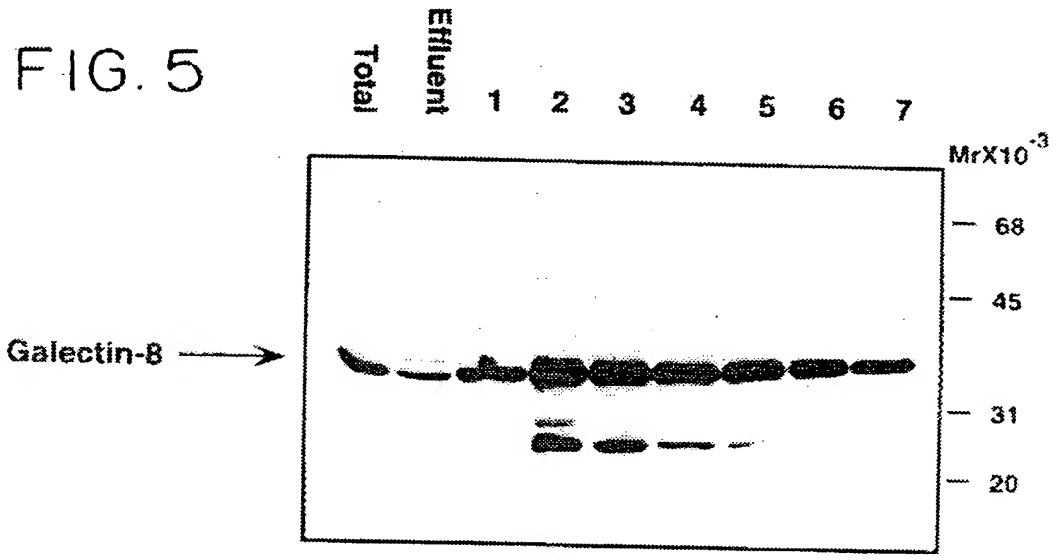


FIG. 6A

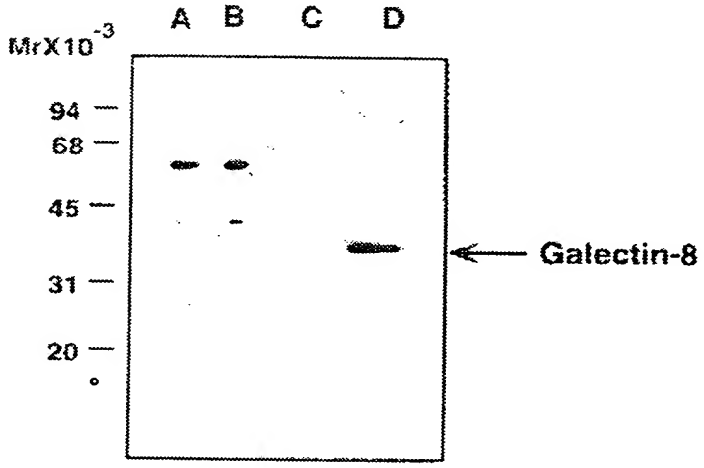


FIG. 6B

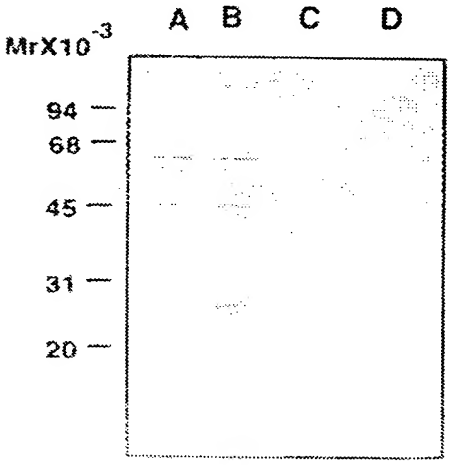


FIG. 7

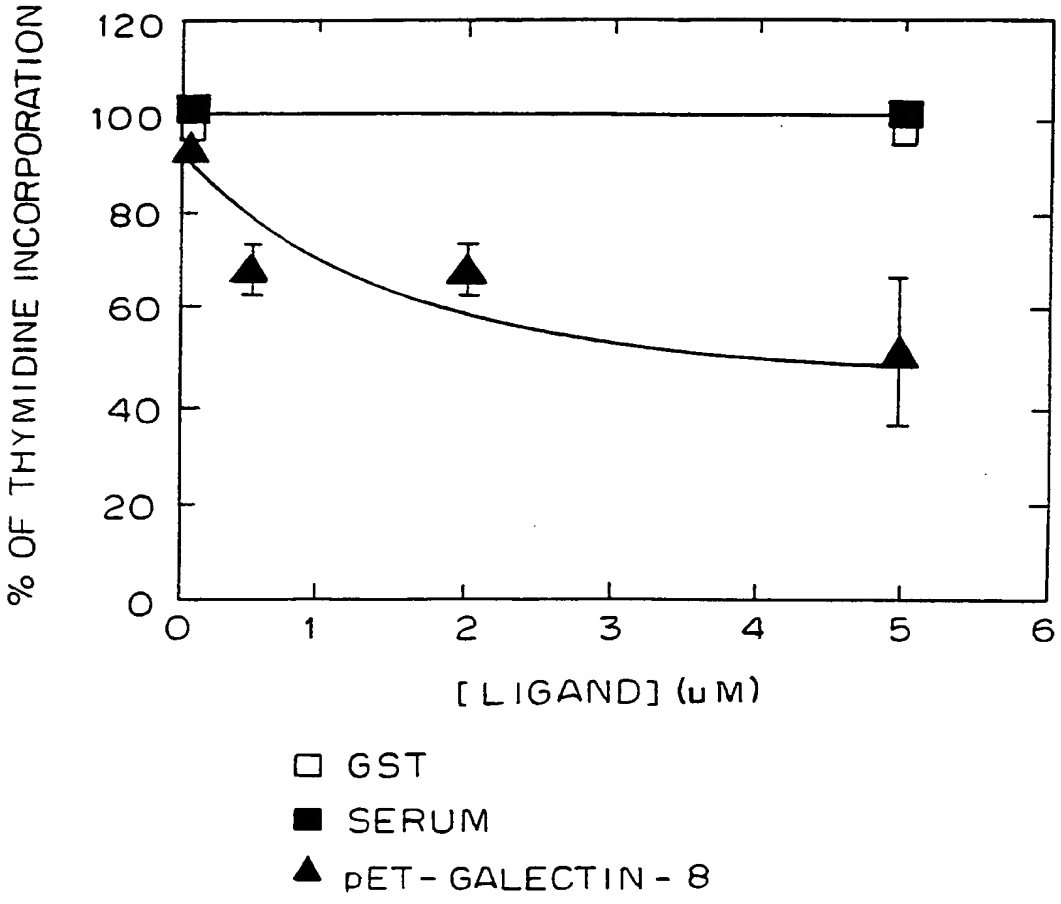
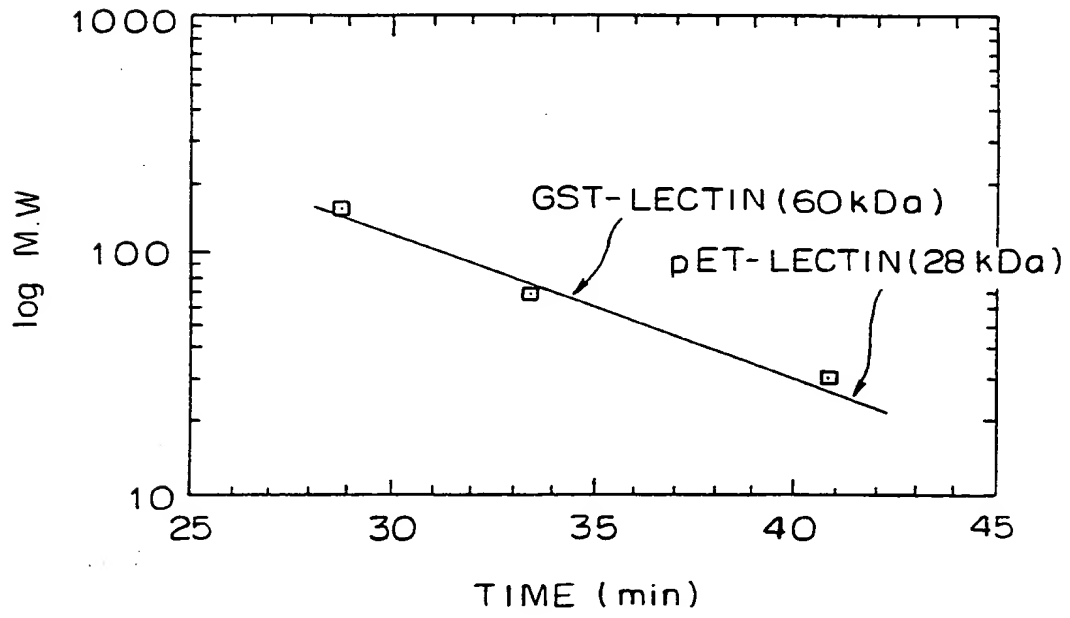


FIG. 8



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13679

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 69.3, 70.1, 70.3, 71.1, 240.1, 320.1; 514/2, 12; 530/300, 350, 387.1, 387.9; 536/23.1, 23.5, 24.31

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS; STN files Biosis, Medline, EMBASE, CA, WPIDS; search terms galectin?, gal(w)lectin?; antibod? [in combination with previous terms] .

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Biological Chemistry, Volume 268, No. 8, issued 15 March 1993, Y. Oda et al, "Soluble Lactose-binding lectin from Rat Intestine with Two Different Carbohydrate-binding Domains in the Same Peptide Chain", pages 5929-5939, see especially pages 5931-5937.	1-13
Y	Biochemistry, Volume 29, No. 35, issued 1990, M. Robertson et al, "Human IgE-Binding Protein: A Soluble Lectin Exhibiting a Highly Conserved Interspecies Sequence and Differential Recognition of IgE Glycoforms", pages 8093-8100, see especially pages 8095-8096.	1-13

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 FEBRUARY 1995	Date of mailing of the international search report 13 MAR 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ARIE M. MICHELSOHN, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13679

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	Journal of Biological Chemistry, Volume 269, issued 8 July 1994, B. Mehul et al, "Structure of Baby Hamster Kidney Carbohydrate-binding Protein CBP30, an S-type Animal Lectin", pages 18250-18258, see especially pages 18252-18255.	1-14
Y,P	Journal of Biological Chemistry, Volume 268, issued 15 December 1993, J. Herrmann et al, "Primary Structure of the Soluble Lactose Binding Lectin L-29 from Rat and Dog and Interaction of Its Non-collagenous Proline-, Glycine-, Tyrosine-rich Sequence with Bacterial and Tissue Collagenase", pages 26704-26711, see especially pages 26705-26708.	1-13
Y,P	Cell, Volume 76, issued 25 February 1994, S. Barondes et al, "Galectins: A Family of Animal beta-Galactosidase-Binding Lectins", pages 597-598, see entire document.	1-13
Y,P	Journal of Biological Chemistry, Volume 269, issued 19 August 1994, S. Barondes et al, "Galectins: Structure and Function of a Large Family of Animal Lectins", pages 20807-20810, see entire document.	1-13
Y	Cancer Research, Volume 49, issued 1 March 1989, R. Lotan et al, "Biochemical and Immunological Characterization of K-1735P Melanoma Galactoside-binding Lectins and Their Modulation by Differentiation Inducers", pages 1261-1268, see the Abstract and especially pages 1263-1264.	14
A	Journal of Biological Chemistry, Volume 263, issued 15 July 1988, K. Drickamer et al, "Two Distinct Classes of Carbohydrate-recognition Domains in Animal Lectins", pages 9557-9560, see entire document.	1-13
Y	Journal of Biological Chemistry, Volume 264, issued 5 February 1989, J. Laing et al, "Biochemical and Immunological Comparisons of Carbohydrate-binding Protein 35 and an IgE-binding Protein", pages 1907-1910, see especially pages 1908-1909.	1-14
A	Journal of Biological Chemistry, Volume 267, issued 15 July 1992, D. Hsu et al, "Biochemical and Biophysical Characterization of Human Recombinant IgE-binding Protein, an S-type Animal Lectin", pages 14167-14174.	1-13

INTE TIONAL SEARCH REPORT

International application No.
PCT/US94/13679

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Biochemistry, Volume 32, issued 1993, S. Massa et al, "L-29, an Endogenous Lectin, Binds to Glycoconjugate Ligands with Positive Cooperativity", pages 260-267.	1-13
Y	Journal of Biological Chemistry, Volume 263, issued 5 May 1988, S. Jia et al, "Carbohydrate Binding Protein 35: Complementary DNA Sequence Reveals Homology with Proteins of the Heterogenous Nuclear RNP", pages 6009-6011, see entire document.	1-13
Y	WO,A,91/08290 (PILLAI ET AL) 13 June 1991, see entire document.	1-14
Y	Cancer Research, Volume 49, issued 01 July 1989, A. Raz et al.,"Identification of the Metastasis-associated, Galactoside-binding Lectin as a Chimeric Gene Product with Homology to an IgE-binding Protein", pages 3489-3493, see entire document.	1-13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13679

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark n Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13679

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 38/00, 38/04, 38/16, 38/17; C07H 21/00, 21/04; C07K 2/00, 14/00, 16/00, 16/18; C12N 5/10, 5/16, 7/01, 15/00, 15/09, 15/10, 15/11, 15/12, 15/63; C12P 1/00, 21/00, 21/02

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 69.3, 70.1, 70.3, 71.1, 240.1, 320.1; 514/2, 12; 530/300, 350, 387.1, 387.9; 536/23.1, 23.5, 24.31

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-13, drawn to nucleic acids, corresponding vectors and host cells, and corresponding proteins and peptides, classified in U.S. Class 435, subclasses 69.1, 240.1, 320.1, Class 530, subclasses 300, 350.

Group II, claim 14, drawn to antibodies, classified in U.S. Class 530, subclass 387.1.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Groups I and II are materially distinct compositions of matter that are distinguished, one from the other, by their special technical features: the polynucleotides and polypeptides of Group I and the antibodies of Group II have materially different structures and functions. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.